

An Unbiased Way to Estimate Total Quantities: The Fractionator Technique

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ABSTRACT

The fractionator is a straightforward and unbiased method to estimate virtually any type of total quantity using sectional profiles of tissue particles. Its basic principle and ease of use make this approach a method of choice in many microscopical quantitative studies. In this review, we will summarize the basic principles of the fractionator approach and give some examples focused especially on some basic topics in neuroscience research.

Key Words: Cell number, fractionator technique, tissue, neuroscience

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Introduction

Determining the total number of particles, along with any total quantity is a challenging task in biological specimens. We generally have to use microscopic sections to investigate the inner structure of biological organs or tissues whereas the sectioning procedure itself is misleading in terms of quantification.

General considerations Profiles and particles

The first and important issue about sectioning is that sectioning generates some profile of the tissue components, such as cells, fibers or organelles. Generated profiles as a result of sectioning do not truly represent the particles which they are generated from, because the

number or the shape those profiles are determined by a number of factors, including sectioning direction, particle size and spatial distribution of particles. Thus, particle profiles seen on sections are not reliable sources for quantification of particles. The most important point to note is that the profiles are not equal to particles, so their number is not necessarily correlated with, or reflect, the number of particles which they are generated from (Fig.1). Historically this problem is well known in the early stages of morphometric studies and there have been some attempts to overcome this discrepancy (Abercrombie, 1946).

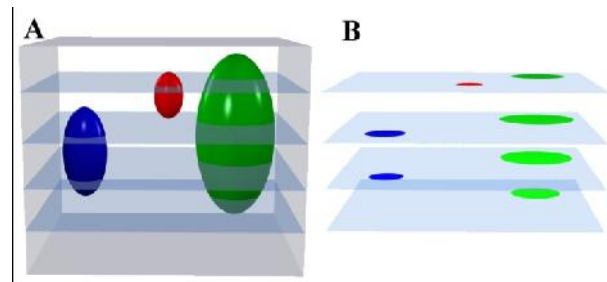


Figure 1. The effects of particle size on profile numbers generated by sectioning procedure are seen. The big sized particle is seen more sections than small one and as it seen on

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the figure, counting each of the profiles on the section would not give the real particle number found in the tissue block.

Section thickness

When dealing with sections it is sometimes intuitive to interpret the sectional images as two-dimensional (flat) images, with no significant depth or thickness. In reality, even in the thinnest possible sections obtained for electron microscopy, the sectional thickness is present and important. Thickness generates important effects on the quantity of the particles as illustrated in **Fig. 2**.

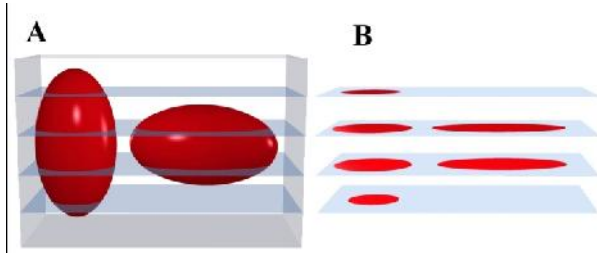


Figure 2. The effects of particle orientation on profile numbers generated by sectioning procedure are seen. Although the particle that is orthogonal to section plane on the left side gives 4 profiles but the horizontally oriented particle gives 2 profiles in the section, although they both have the same size.

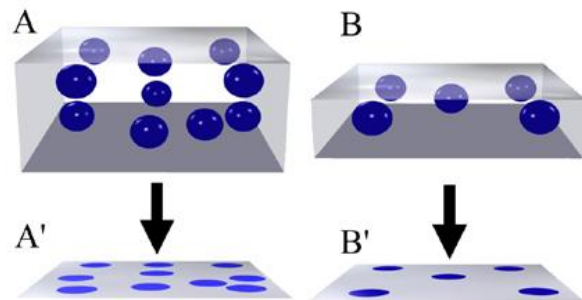


Figure 3. Relationship between section thickness and number of particle profiles in the image of section. Increasing the thickness will increase the number of particle profiles seen in the section. It is a misleading factor when such sections are interpreted as flat images without any depth.

Sections and dimensions

Another issue is the dimensional reduction problem. All sectioning procedures generate sectional profiles of the tissue components with a dimension decrease. As illustrated in **Figure 3**, all tissue components appear on sections as profiles having minus 1 dimension then they are seen in 3D. A line or curve (1D) component gives a point (1-D) profile, while a plane of surface (2D) component ends up with a curve or line (1D) and a three dimensional object with a volume gives a planar (2D) profile. Such a change may easily be confused the quantitative estimations derived from

sectional images. This problem is generally known as the “dimensional reduction problem” (**Fig. 4**).

Determining the particle number “Number” in the sections

After seeing the dimensional reduction problem, it would be necessary to review the concept of “number” in the light of the topics that we have listed so far. Number is a parameter which does not depend on the dimensional profiles of the objects. Every object is equal in terms of number, no matter how big or small, how complicated or abstract they are. Thus, the “number” is generally considered as a “zero-dimensional parameter”. As you can recall from the dimensional reduction problem, sections cannot supply any information about the number of tissue components; since the number, as a zero dimensional quantity, cannot be represented on sections (due to the dimensional reduction). Anyone who seeks the number of particles must use the regular sections in a way which they can create a three dimensional information system. And this is what the most of the modern design-based unbiased stereological methods are all about (Gundersen, 1977; Mayhew and Gundersen, 1996; Oorschot, 1994; Weibel, 1969). To overcome such obstacles, the disector method is a very elegant way to gather quantitative information from sections; as described previously. The disector application gives us the density of the particles in a unit volume (Sterio, 1984, Tümkaya *et al.*, 2010). But in most of the cases we face in biology, such a parameter is not adequate, no matter how carefully derived. Since the volumetric changes are hidden in such an approach, density changes do not tell much about the total differences in number, so we need to proceed to a suitable method to estimate the total number of tissue components, independent from the orientation or distribution of the particles as well as volume change (West, 1993). The fractionator method is the solution of this problem.

The Fractionator

Maybe the simplest and the most straightforward one among all the stereological methods is the fractionator. It is a very strong and simple means to achieve a total quantity from a relatively small set of samples obtained from a structure of interest.



Its strength largely originates from its simplicity. When applied according to its simple set of rules, it is not known how to generate a false or biased estimate using the fractionator. This is principally due to the fact that the fractionator estimates are not affected by tissue processing artifacts, tissue distortions including swelling or shrinking or any structural alterations naturally occur during the sectioning process itself. It also requires no assumptions about the size, shape and the orientation of the particles of interest (Gundersen *et al.*, 1988a; Gundersen and Jensen, 1987). Such a methodology is very precious for investigators who seek for a simple, straightforward and unbiased alternative for a rather complicated subject like total quantity estimations in biological tissues.

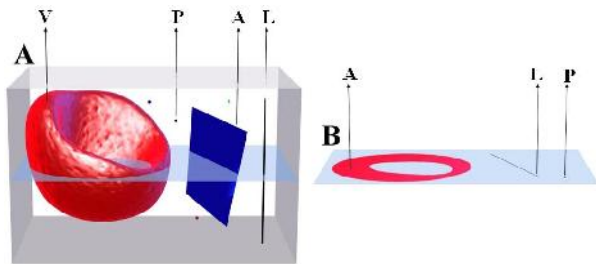


Figure 4. The relationship of dimensions in 3D to those seen on the sections. When cut by a two-dimensional sectional plane, a volume (V) gives an areal (A) profile; an area (A) appears as a linear profile (L), a linear (L) feature becomes a point (P) while point-like properties yield no detectable trace on sections.

The basic logic of the fractionator technique can be summarized as follows:

- Take any structure with known boundaries and limits under investigation.
- Divide it into approximately even pieces (sections, slabs or alike).
- Take a systematic and random sample from the whole set, giving an equal probability of each point of the structure for being selected for the final sample (if necessary, repeat such sampling steps in any desired number of steps or sub-samplings).
- Note the fraction of your final sample compared to the whole, namely the sampling fraction.
- Count or measure the parameter of interest on the final sample.

- Multiply your results with the reciprocal of your sampling fraction, and then you have the total quantity of interest for your particular structure or organ.

This simple sampling regime is called “the fractionators” in modern, design-based stereology and can be applied in a wide variety of quantitative problems in morphometry (Gundersen *et al.*, 1988a; West and Gundersen, 1990; West *et al.*, 1991; Rasmussen *et al.*, 1996; West, 1996; Howard and Reed, 1998; Schmitz and Hof, 2000; Şahin *et al.*, 2009).

A Schematic illustration

We can use the illustration in **Fig. 5** to demonstrate the unbiasedness of the fractionator principle. In this illustration there are some particles contained in an area (or in a confined space) marked with an elliptic border. The total number of these particles is actually 182 (note that such a real value may not be available in a real particle number study).

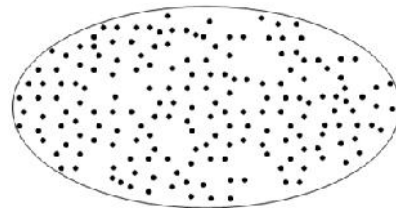


Figure 5. In this illustration there are some particles contained in the area that is marked with an elliptic border. The total number of these particles is actually 182.

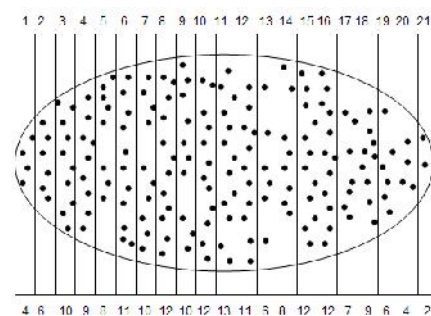


Figure 6. The whole structure is divided into evenly spaced pieces (or sections). These illustrate parallel sectioning procedure. The upper row of numbers shows the number of pieces from 1 to 21 and the bottom row of number shows the number of particles in the respective section.

Let’s assume that we want to estimate the total number of particles in this example, without the knowledge of the actual number. We can



use the fractionator sampling scheme for this purpose rather than counting all the particles exhaustively (which is generally impossible for a typical biological study). First, we divide the whole structure into evenly spaced pieces (or sections). **Fig. 6** illustrates this parallel sectioning procedure. The upper row of numbers in **Fig. 6** shows the number of pieces from 1 to 21 and the numbers at the bottom are the number of particles in the respective section.

To perform the estimation, we should choose a set of samples from our structure. Let's say we have determined a sampling interval of 1/3; which means that we will choose a random piece within the first 3 and then continue to select every third piece after the first sampled piece (Systematic Random Sampling). After this sampling procedure, we count all the particles contained in the final sample. If we count all the particles in the final sample and multiply it by 3, we can estimate the total number in the whole structure. If we have chosen the second piece as the first sample, our estimation process will be as follows:

Sampled section #	Number of particles
2	6
5	8
8	12
11	13
14	8
17	7
20	4
Total	58

Sampled section #	Number of particles	Sampled section #	Number of particles
1	4	3	10
4	9	6	11
7	10	9	10
10	12	12	11
13	6	15	12
16	12	18	9
19	6	21	2
Total	59	Total	65
Estimated total number of particle	177	Estimated total number of particle	195

If we multiply the total number (58) by 3, we get an estimate of 174 as total number of

particles. As can be seen from the above example we have a different estimate than the real value, 182. Unbiasedness means that we have to use a denser sampling scheme, than used previously approach the real value. Let's proceed on our example to clarify this concept. The next step would be to consider other possibilities which we could encounter in our sampling procedure. We can look at the same situation when we select the first and the third section as the first sample for our systematic random sampling scheme. In this case, our estimation will be as follows (check also the **Figure 5**).

As you can see, we still have different values (177 and 195) from the real particle number (182) after each estimate. But, if we calculate the mean value obtained from these three estimates:

$$\text{Estimated mean particle number} = (174+177+195)/3 = \underline{182}$$

As can be seen from the result, an exhaustive sampling of the whole structure enables us to achieve the true value. If our estimation method introduces bias in a way or another, we could not expect to see such a result.

Application of physical and optical fractionator

The physical fractionator

Disector is the most frequently used counting method in stereology. Thus, when we consider the two basic types of disector, namely the physical and optical disector, fractionator schema can be classified by the type of counting method employed during the procedure. If the final particle number estimate is done by physical disectors, the method should be called "physical fractionator", whereas the use of optical disectors in the final estimates means that the method should be called "optical fractionator". Historically the physical disector is older than the optical disector since it became available earlier (Gundersen *et al.*, 1988b; West and Gundersen, 1990; West *et al.*, 1991).

General rules of the physical fractionator

Currently the physical fractionator is generally used for electron microscopic studies (where an adjustable focal plane is not available) and to count particles that could not be wholly



contained in a single thick section (or slice). The general steps of physical fractionator can be summarized as follows (Sterio, 1984; West, 1999):

1. If the organ of interest is not suitable for direct sectioning for microscopy, it must be cut into similar-sized pieces using a suitable method. Although this cutting procedure can be done arbitrarily, cutting in parallel directions with an effort to generate pieces in approximately similar in size would increase the efficiency and precision of the study.
2. A systematic random selection of pieces can be chosen according to the optimum sampling interval determined by a previous pilot study. This first sampling fraction (f_1) must be noted.
3. If the pieces cut from the organ are still large for microscopic investigation, steps 1 and 2 can be applied on the selected subsamples of the organ as needed. If there will be any more sampling in this step, it should be done by systematic random sampling and the sampling fraction must be noted (f_2).

Final SR samples of the organ then will be processed for microscopic investigation and cut into sections with a suitable equipment. In most cases we need to make another set of SR samples from the section arrays generated from this cutting procedure. This step must also be note as a new sampling fraction (f_3). Since we employ the physical disectors for the counting of particles, it is important to choose every section with its consecutive section in order to generate a “disector pair” (Sterio, 1984; Bradley *et al.*, 1994).

4. Corresponding counting areas must be carefully registered for disector pairs and disector counting must be carried out according to the rules described earlier. (See the Physical Disector). During this counting procedure the area of the unbiased counting frame ($A_{(f)}$) and the step size that used to meander the sectional profile (A_{step}) should be known to estimate the final sampling fraction ($f_4=A(f)/A_{\text{step}}$).
5. Total number of particles in the whole organ (N) can then be estimated by multiplying the number of particles

counted (Q^-) by the reciprocals of the previous sampling fractions:

$$N = \sum Q^- \cdot (1/f_1) \cdot (1/f_2) \cdot (1/f_3) \cdot (1/f_4)$$

(Eq. 1)

The Optical Fractionator

Optical fractionator differs from physical fractionator only by the final counting method. In this case optical disectors are used for particle counting instead of physical disector. Optical fractionator approach is the most frequently used method in particle number estimations, especially in neurosciences (Pakkenberg and Gundersen, 1988; Pakkenberg and Gundersen, 1997; Schmitz, 1998; Şahin *et al.*, 2009).

Now we will summarize a real life example to describe a typical optical fractionator application.

An Example

Estimation of the total neuron number in chick (gallus domesticus) hippocampus

Hippocampus is an important brain region for learning and memory in avian species, as well as in the mammals. It is important to know the total neuron number for developmental and pathophysiological studies concerning this are. Thus, an unbiased estimation of the total hippocampal neuron number is necessary for our investigation. In this example, 5 newly hatched Broiler chicks are used for the estimation procedure.

Animals were perfused intracardially with physiological saline followed by 4% neutral formalin under deep ether anesthesia. Following the perfusion, brains were removed as a whole and left in the same fixative solution for 3-5 days. After this post-fixation, all brains were individually embedded in paraffin blocks after routine tissue processing procedures. Tissues in paraffin blocks attached to the microtome block holder in a position suitable to obtain horizontal serial sections of hippocampal area. All brains were sectioned in dorsoventral direction with a thickness of 40µm. According to a pilot study, it has been decided to sample every fourth section with a random start between the first four sections. This sampling step has been recorded as “section sampling fraction” (SSF=1/4). Sampled sections then mounted on glass slides and stained with Cresyl violet staining (**Fig.7**).



According to the pilot study a step size of $300\ \mu\text{m} \times 300\ \mu\text{m} = 90000\ \mu\text{m}^2$ has been determined for meander scanning of all hippocampal sectional fields (**Fig. 1**). Beginning from the first section containing the hippocampus all sections were scanned exhaustively using this step size. In each step, an unbiased counting frame of $400\ \mu\text{m}^2$ was used for particle counting (**Fig. 7-9**). Thus, an area sampling fraction of $400/90000$ was applied in each field. This value was also recorded as “area sampling fraction” ($\text{ASF} = 400/90000$).

Each disector probe is actually a virtual counting box located in every counting field and its height is less than the section thickness. This means that in every counting field, a known fraction of section thickness (disector height/section thickness) was chosen for particle counting. For the present study, the height of the optical disectors was determined to be $20\ \mu\text{m}$ and the mean section thickness (t) was estimated for each individual (For example for #1, $t=39.73\ \mu\text{m}$). In this case there is a third sampling step which is called “thickness sampling fraction” ($\text{TSF} = 20/\text{mean section thickness for each animal}$).

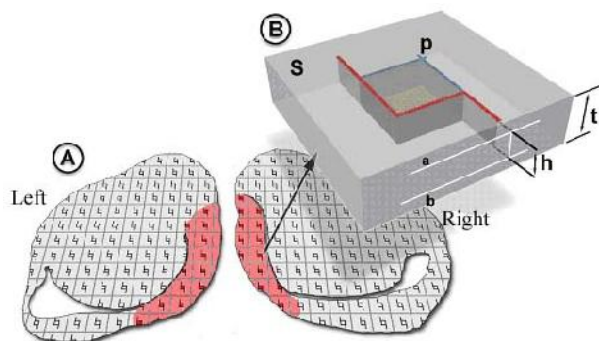


Figure 8. Schematic representation of counting areas, counting frames and disector probe used in this optical fractionator study. Red color areas in (A) are horizontally sectioned hippocampal profiles. All sectional profile of the brain was divided into systematic and randomly positioned stepping areas while only the areas hitting the hippocampal profiles were selected for stereological analysis. Unbiased counting frames located in each step are also depicted. A single disector probe is depicted in (B) where “ t ” is the section thickness; “ h ” is the disector height; “ a ” and “ b ” are upper and lower guard zones respectively and “ p ” represents the fixed point used for sampling reference in each step. If this point “ p ” hits the hippocampal tissue, this step is included in the counting procedure (Courtesy of Dr. Aslan).

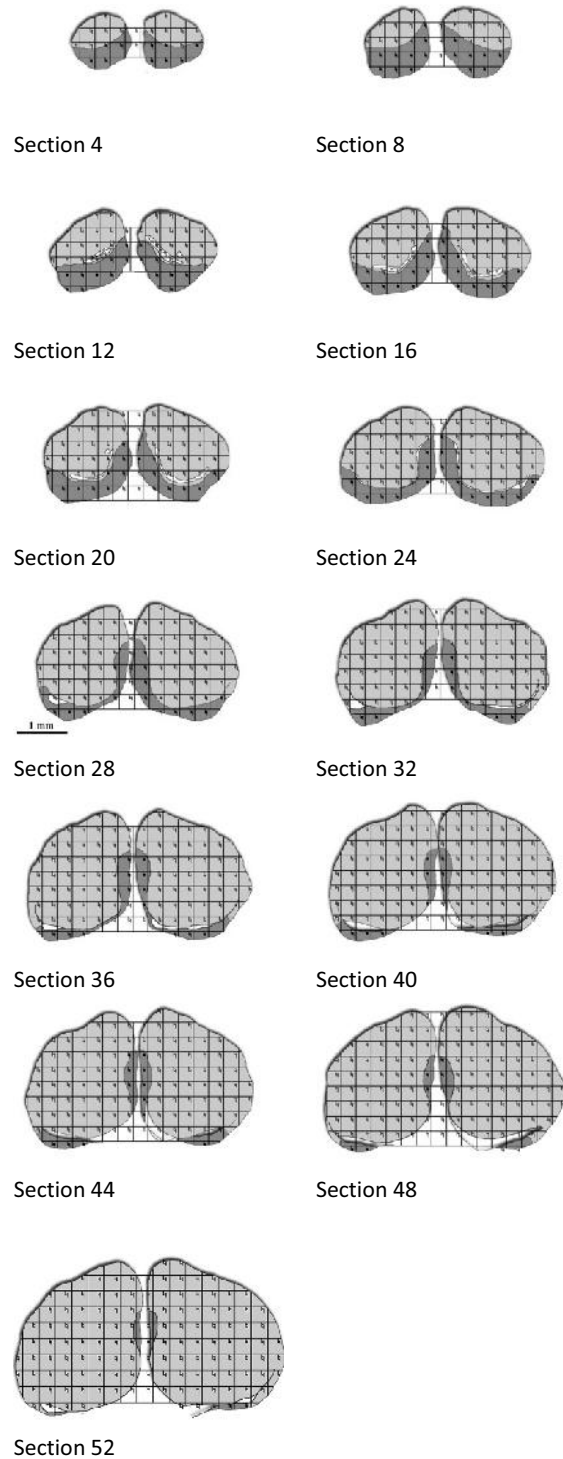


Figure 7. Systematic random samples (1/4) from a serial sectioning of the chick hippocampal formation. Boundaries of brain and the hippocampus were drawn using a camera Lucida (Nikon 105). Areas with darker shade are hippocampal profiles. Square grids located on the sections represent the meandering steps across sections and the smaller areas indicated in each square represents the location of the unbiased counting frame in each step. The proportions depicted here are not actual and drawn only for demonstration purposes (Courtesy of Dr. Aslan).



All those sampling steps in the section level are done with the help of a computer assisted stereological workstation (Olympus CAST Grid, Denmark). The last phase is to

count particles using optical disectors. Please note that the counting procedure must be consistently applied to all fields included in this study.

Application of the optical disector

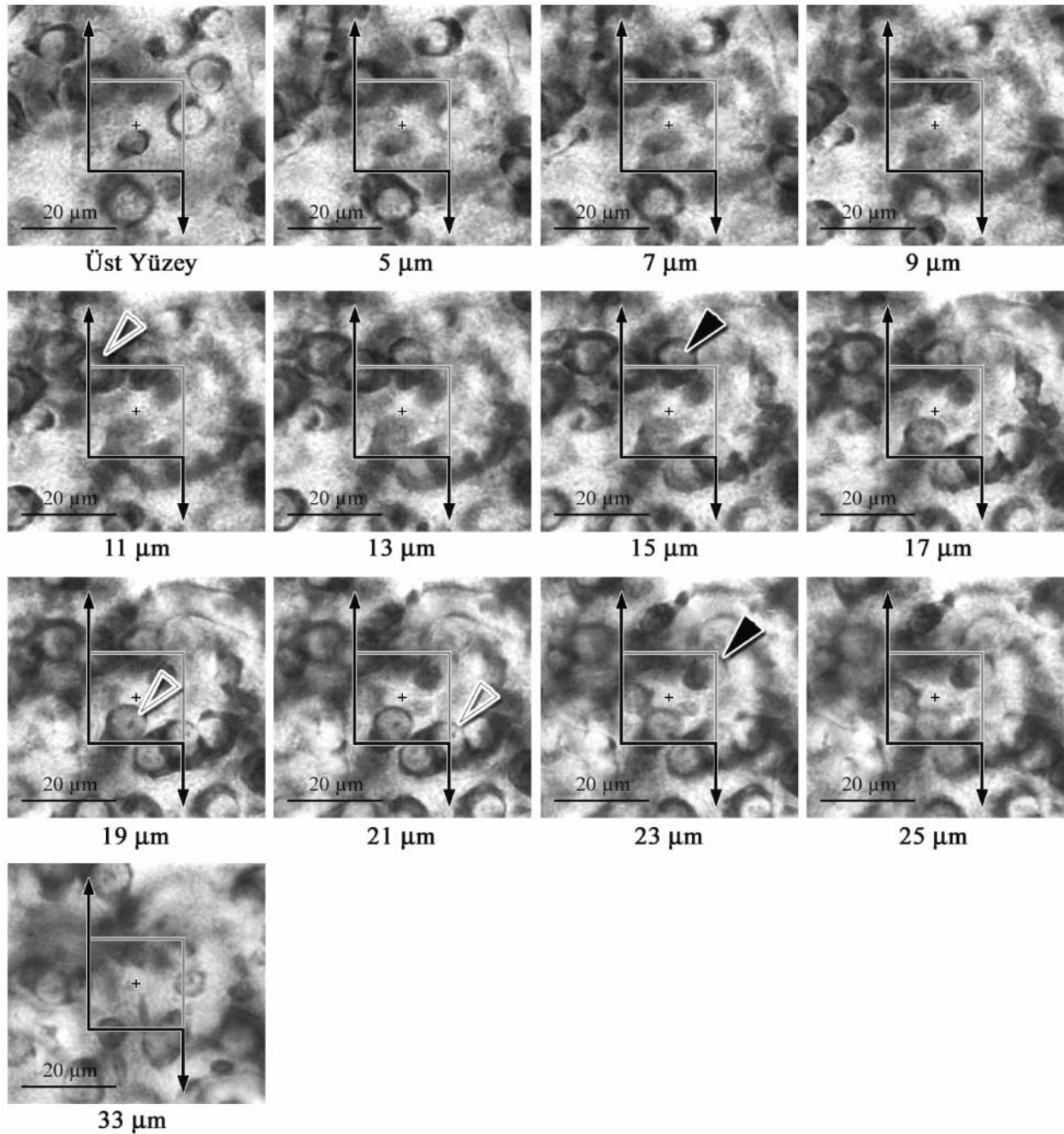


Figure 9. A sample counting procedure: The first nucleus visible in the frame (light edged arrowhead at 11 μ m) is not counted because it hits the forbidden line. The nucleus profile at 15 μ m is counted since it is in contact with the inclusion line of the counting frame. Nuclei at the 19th μ m and the 21th μ m are excluded due to the contact with forbidden line. Nucleus at the 23rd μ m (black arrowhead) is counted. The level of 25 μ m is the end of our disector probe and the rest of the section will be left as the lower guard zone. Particles contained in this area will not be counted. The last clear view of the section during a downward movement represents the level of the lowermost surface. Thus we can see a 33 μ m distance from the upper and the lower surfaces of the section. In this samples probe, we have counted 2 disector particles (neuronal nuclei). A similar approach must be applied to all sectional fields of the hippocampus to obtain the total neuron number for this particular animal (*Courtesy of Dr. Aslan*).



Values obtained from the counting described above are presented in the following table:

Table 1. Hippocampal nerve cell counts using optical disector.					
Animal number	1	2	3	4	5
Mean section thickness (μm)	39.73	34.93	33.67	37.87	35.53
Step length (μm)	300	300	300	300	300
Total number of points	166	209	210	192	174
h(dis) (μm)	20	20	20	20	20
a(step) (μm^2)	90000	90000	90000	90000	90000
a(frame) (μm^2)	400	400	400	400	400
Number of disector particles	441	494	490	463	512
1/ASF [$a(\text{step})/a(\text{frame})$]	90000/400	90000/400	90000/400	90000/400	90000/400
1/TSF [t/h]	39.73/20	34.93/20	33.67/20	37.87/20	35.53/20
1/SSF	4	4	4	4	4
Total cell number (N)	788441	776493	742423	789021	818611

To estimate the total number of hippocampal neurons, we multiply the total counted disector particles (ΣQ) by the reciprocals of the each sampling fraction. This estimation is only for the animal #1 and the same approach must be applied to all other animals in the group:

$$N_1 = \Sigma Q \cdot (1/SSF) \cdot (1/ASF) \cdot (1/TSF)$$

$$N_1 = 441 \cdot 4 \cdot (90000/400) \cdot (39.73/20)$$

$$N_1 = 788441.$$

Estimation of the reference volume during the optical fractionator application

A prominent advantage of the fractionator technique is that we do not need to know the reference volume since this approach depends on direct estimation of total number of particles. But in some studies there may be an additional need to know the changes in reference volume of an organ. There is a very simple way to achieve this in the studies of fractionator. One can easily estimate the volume of a structure during a fractionator estimate by simple counting every sampling point hitting the tissue of interest. Point-counting or Cavalieri's Volume Estimator is a widely used method for estimating the volume of sectioned material (Sahin *et al.*, 2001; Elfaki, 2011). Since the sampling point itself represents an area equal to the step size used for meander scanning of the section plane, multiplying the total number of points by the step size can give an unbiased estimate the reference volume of that structure (Howard and Reed, 1998).

To estimate the reference volume using point-counting, it is necessary to determine a fixed point in or on the unbiased counting frame used in particle counting. In our sample study, this fixed point located at the center of the counting frame. All points hitting the profiles of the tissue of interest (in this case, the hippocampus) must be counted through all sampled sections. This process is actually equal to throw an equally-spaced point grid onto the sectional profiles with an interval equal to the step size (**Fig. 3**). As a result, if we multiply the total number of points (ΣP) across all sections from an individual by the stepping size (point-associated area; [$\mathbf{a}(\mathbf{p})$]), we can estimate the total profile area of the hippocampus visualized from the sampled sections. Multiplying this result with mean section thickness (t_{mean}) gives us an unbiased estimate of the reference volume (V_{ref}) of the hippocampus:

$$V_{\text{ref}} = \Sigma P \cdot \mathbf{a}(\mathbf{p}) \cdot t_{\text{mean}}.$$

Appendix I

Estimation of section thickness in optical fractionator studies

Section thickness information is essential for optical fractionator studies in order to measure the some necessary parameters including disector height and thickness sampling fraction. The most frequently used method for section thickness measurement is done by measuring the travel distance of focal plane by measuring the vertical displacements of the microscope stage. We thus need a special equipment to enable measuring the vertical (z -axis) displacements of the



microscope stage in micrometric range. It is necessary because any vertical focal plane displacement through the thickness of a thick section requires an opposite movement of the microscope stage (when the microscope stage moves up by the turning of the screw, focal plane travels down deeper into the section). If we can measure the amount of the stage displacement with sensitive equipment, we will have the chance to measure any two points located in different depths of a thick section.

Some gadgets for the measurement of stage movements

The best and the most precise way of measuring the stage displacement are to use a digital or analog microcator. Microcators consist of a sensitive measuring apparatus (having 1-1/10 μ m sensitivity) attached to any movable part of the microscope stage and a digital or analog dial that displays the amount of movement in z-axis. Especially the digital microcators are generally a bit expensive and could not be found any in every laboratory readily. Another alternative would be to use analog microcators, which can be manufactured using industrial calipers available elsewhere (Korkmaz and Tmkaya, 1997; Kaplan *et al.*, 2001; Kaplan *et al.*, 2005).

Measurement of section thickness in light microscopy; movable focal plane method

After the attachment of suitable equipments for the measurement of movements of the microscope stage, section thickness can be measured by the movable focal plane method as follows (Howard and Reed, 1998):

1. Put your specimen on the stage and generate a clear and sharp view by focusing. After getting a fine view lower the stage with the knob until you get a blurred vision.
2. Blurred vision indicates that the focal plane is above the upper surface of the section. From this point, reverse the direction of movement (by raising the microscope stage), until the point of first clear vision of tissue texture. This point corresponds to the upper surface of the section and you can reset your microcator to "zero" at this point (alternatively, note the reading of

your equipment and consider it as the "zero" level).

3. After resetting, continue your movement by raising the stage (going deeper into the section) slowly. When the focal plane leaves the section from the lower surface, another blurred view will be seen.
4. Relocate this last clearly seen level on your microscope and check your microcators reading. The value given in the reading (or the difference between the first and the last values) will give you the section thickness.

The precision of the measurement described above is dependent on the basic microscope setup as well as the sensitivity of the reading equipment. For example, if we use an immersion objective for measurement, we definitely should use immersion oil for a correct measurement of section thickness. There is also some degree of inter-observer variation in such measurements but studies show that it is generally a minute difference for a typical study and can be ignored. Of course, a little practice and familiarity will increase the stability and the degree of precision.

Estimation of the mean section thickness

The method described above was about the measurement of thickness in a single point of view on a microscopic section. However, since there is a considerable variability in terms of section thickness between the different parts of a single section, we generally prefer to use the mean section thickness estimated from the measurements gathered from different points of a section. As stated previously, a sectional area is generally divided into a series of equally-spaced steps and scanned through these steps exhaustively for a typical stereological study. If we prefer to measure the section thickness in every step (or in systematic steps sampled in a systematic-random fashion), we can take average these measurements to estimate a mean section thickness for this particular section. Similarly, averaging the mean thickness estimations across a set of sections obtained from a single individual will give us an overall mean section thickness for that animal (or subject). For example while it was sampled the whole area



in the sampled sections obtained from a chick brain for neuron counting, we generally preferred to measure the section thickness in every 10th step (with a random start among the first ten meandering steps) and average all measurements after the section set has been analyzed exhaustively. Thus we obtain an unbiased estimate of the mean section thickness for that individual (Howard and Reed, 1998).

Estimating the degree of error for a subject and a group estimation

An important feature of stereological methods is being independent of erroneous assumptions that could lead systematic deviation from the true value of the quantity under investigation. This is generally known as “unbiasedness”. Another important feature is that stereological methods enable us to see the variability due to the design of the overall study. Independent from the nature of the method we use, they will always be variability due to the statistical nature of our studies. It is important to keep this natural variability in acceptable ranges for the validity of the results.

Estimation of coefficient of error (CE) value for the stereological studies has been described previously (for details, see refs: Gundersen, 1986; Gundersen and Jensen, 1987; Schmitz, 1998; Gundersen and Jensen, 1999; Schmitz and Hof, 2000). CE is a quantitative measure of the precision of the stereological design apart from the inter-individual variability which depends on biological differences between individuals. The next step would be to estimate the coefficient of variation (CV) for each group included in the study which is an indicator of biological variability among individuals (Gundersen, 1986; Gundersen *et al.*, 1999).

Coefficient of error

Estimation of CE is made simply by **Eq 2**. On the other hand if one need a detail estimation of CE, it should be used other estimation approach that is shown in below.

$$CE \cong \frac{1}{\sqrt{n}} \text{ (Eq. 2)}$$

An example of estimation of CE for one subject (Gundersen, 1986; Gundersen *et al.*, 1999).

Animal #1				
Section #	Q	Q _i ·Q _i	Q _i ·Q _{i+1}	Q _i ·Q _{i+2}
1	45	2025	2745	2610
2	61	3721	3538	3904
3	58	3364	3712	2842
4	64	4096	3136	1664
5	49	2401	1274	1764
6	26	676	936	702
7	36	1296	972	864
8	27	729	648	486
9	24	576	432	360
10	18	324	270	90
11	15	225	75	75
12	5	25	25	30
13	5	25	30	10
14	6	36	12	0
15	2	4	0	0
441		Noise:		441
A:	19523	VAR(srs):		118.92
B:	17805	Total Var:		559.92
C:	15401	CE		0.05
		% Noise		78.76

$$A = \sum_{i=1}^m Q_i \cdot Q_i \quad B = \sum_{i=1}^{m-1} Q_i \cdot Q_{i+1} \quad C = \sum_{i=1}^{m-2} Q_i \cdot Q_{i+2}$$

$$CE(Q) = \frac{\sqrt{(3A + C - 4B)/12}}{\sum Q}$$

$$CE(\sum Q) = \frac{\sqrt{\text{Total}_{\text{var}}}}{\sum Q}$$

$$\text{Total}_{\text{var}} = \text{Noise}_{\text{var}} + \text{SRS}_{\text{var}}$$



$$Noise_{var} = \sum Q = 441$$

$$\begin{aligned} SRS_{var} &= (3 \cdot (A - Noise) - 4 \cdot B + C) / 12 \\ &= (3 \cdot (19523 - 441) - 4 \cdot 17805 + 15401) / 12 \\ &= 118.9167 \end{aligned}$$

$$\begin{aligned} Total_{var} &= Noise_{var} + SRS_{var} \\ &= 441 + 118.916 \\ &= 559.916 \end{aligned}$$

$$\begin{aligned} CE(\sum Q) &= \frac{\sqrt{Total_{var}}}{\sum Q} \\ &= \frac{\sqrt{559.916}}{441} \\ &= 0.05 \end{aligned}$$

Coefficient of variation

$$CV = \frac{\sqrt{\frac{\text{Standart Deviation}}{n-1}}}{\sum Q} = \frac{\sqrt{\frac{\sum (X - \bar{X})^2}{n-1}}}{\sum Q}$$

n= animal number in the group

1-day old	N	(X-X _{mean})	(X-X _{mean}) ²
Chick-1	789831	6521	42528657
Chick-2	778050	-5259	27663392
Chick-3	740880	-42429	1800270956
Chick-4	787563	4253	18091411
Chick-5	820224	36914	1362672927
X _{mean} :	783309		CV =0.033

The upper limit for CE and CV is 0.05 and 0.10, respectively. Values below these limits indicate that the study has a sufficient precision for a typical stereological design.

Conclusions

The fractionator's principle is a very simple and elegant way to achieve the total number of particles.

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