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VIRTUAL TEST SYSTEMS FOR GLOBAL SPATIAL SAMPLING IN THICK, ARBITRARILY ORIENTATED UNIFORM, RANDOM SECTIONS.

Jytte Overgaard Larsen[1,2,3] and Hans Jørgen G. Gundersen[1]

[1] Stereological Research Laboratory, Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark, [2] Institute of Pathology, Aalborg Hospital, and [3] Center for Sensory-Motor Interaction, Aalborg University, Denmark.

ABSTRACT

Virtual test systems are test systems where the randomization of the stereological probes is made within volume probes of arbitrarily orientated thick uniform, random physical sections. Complete isotropy and 2D uniform, random sampling in the thick section of the virtual test system is performed by a computer. Subsequent sampling is performed by superimposing a computer generated 2D representation of the test system onto live video images of shallow focal planes within the uniformly, random volume probe. The 2D representation of the test system moves across the computer screen during focal plane displacement and dynamically maps the test system that exists in 3D within the volume probe. Test systems based on isotropic lines in 3D are visualized as moving points in the 2D focal plane, and test systems based on isotropic planes in 3D are visualized as moving lines. In principle only the computer power and our imagination are limits for the design of virtual test systems. Test systems that are isotropic in 3D allow for sampling inside a thick, arbitrarily orientated uniform, random physical section (or at worst vertical sections for local estimators and spatial distributions, when the structure is incompletely contained within the thick physical section), thereby reducing considerably the problems associated with estimation under anisotropic conditions. Practical implementation of the principle are exemplified with estimators of length density and total global length. The major practical advantage of this principle is that for most orientation dependent estimators there is complete freedom to chose the most convenient sectioning direction, and that isotropic sections, in principle, are no longer required for any estimator.

Keywords: Anisotropy, fractionator, isotropic virtual line, isotropic virtual plane, length density, total length.

INTRODUCTION

Design based global length and surface estimators as well as all local estimators require random rotation of the test system with respect to the examined structure (Hennig 1963, Gundersen 1979, Mattfeldt and Mall 1984, Gundersen 1988, Gokhale 1990, Cruz-Orive and Howard 1991, Jensen and Gundersen 1993). Until recently this requirement has been met by

rotating the tissue specimen prior to sectioning to produce either physical *I*sotropic, *U*niformly, *R*andom (***IUR***) (Mattfeldt *et al.* 1990, Nyengaard and Gundersen 1992) or *V*ertical, *U*niformly *R*andom (***VUR***) (Baddeley *et al.* 1986) sections. Physical ***IUR*** or ***VUR*** sections have been unpopular for many reasons. In some tissues a specific sectioning direction is required for recognizing and delineating the region of interest with certainty and the generation of physical isotropic sections may be time consuming and difficult. Moreover, it is particularly elaborate to probe the specimen with many different sectioning directions in order to avoid a high estimator variance in the cases where the examined structure possesses strong anisotropy. Virtual test systems that are randomized within a volume probe of the examined reference space circumvents the use of physical ***IUR*** or ***VUR*** sections (Larsen *et al.* 1997, 1998, Kubínová and Janáček 1998).

VIRTUAL TEST SYSTEMS

Imaging techniques which yield 3D volume information possess freedom to contain a test system with 3Dx4π freedom. However, the volume information is usually obtained from successive 2D focal planes. A stereological test probe with 3Dx4π freedom is now only observed at its intersection with the focal plane, and consequently a line probe is seen as a point in the focal plane, and a plane probe is seen as a line in the focal plane. The position of the point respectively the line changes during focal plane displacement. In live video images of the focal planes the 2D representation of the test system can be visualized through a computer-video interface, and focal plane displacement recordings can be used as feedback information to control dynamically coordinated visualization of the 2D representation of the test system during focal plane displacement, see Fig. 1.

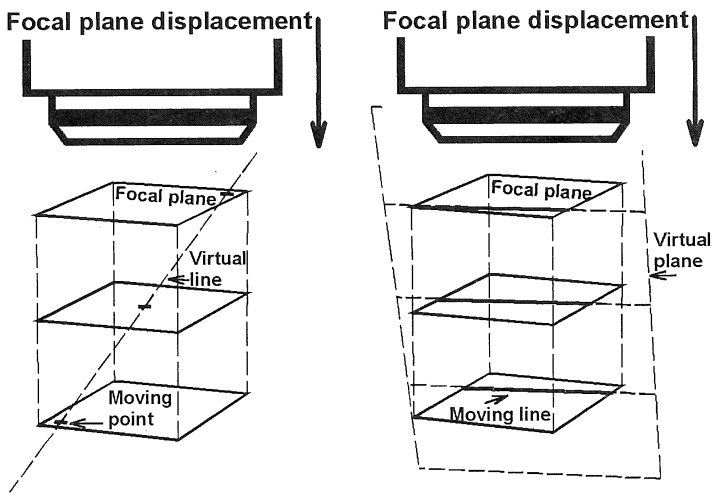

Figure 1

Virtual test probes with 3Dx4π freedom inside a volume probe.

Left *A virtual line probe. The intersection between the focal plane and a linear probe is a point in the 2D focal plane, the point moves across the video image as the focal plane changes.*

Right *A virtual plane probe. The intersection between the focal plane and a planar probe is a line in the 2D focal plane, the line moves across the video image as the focal plane changes.*

ISOTROPIC VIRTUAL PLANES

Software-randomized isotropic virtual planes are implemented in the CAST-grid software, (The International Stereology Center at Olympus Denmark). Live video images of the microscope field are displayed on a computer screen onto which the stereological test system is superimposed through a computer-video interface. The computer receives information about z-axis displacement from the microcator as the focal x-y-plane is moved through the thick physical section. The virtual plane is visualized on the screen as a random line that moves across the screen as the focal plane is moved through the section, the speed and direction of the line displacement depends on the angle of the virtual plane with respect to the focal plane. The orientation of the IUR virtual plane probe is computer selected as illustrated in Fig. 2.

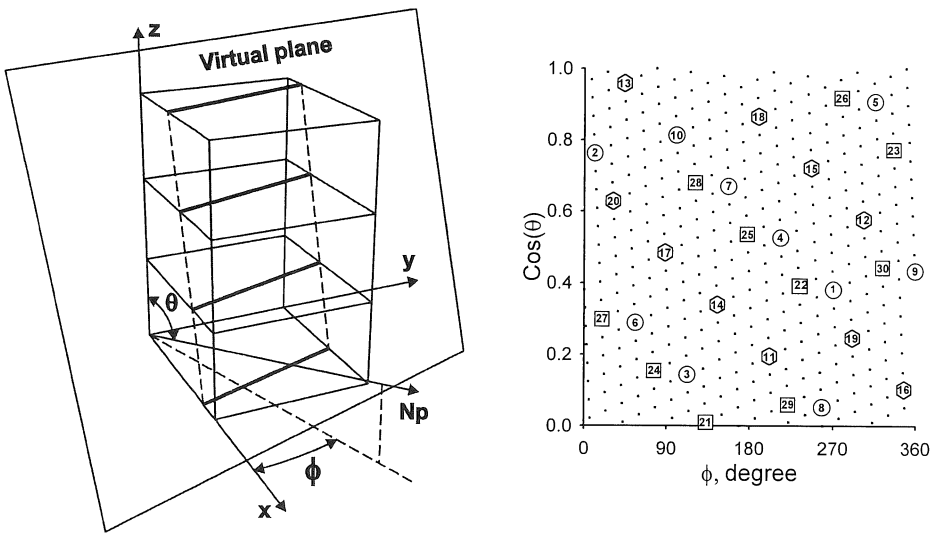

Figure 2

Sampling of systematic, isotropic directions of the normal to a virtual plane, N_p.

Left *Let the direction of focal plane displacement be the z-axis, and the focal plane the x, y-plane. The computer selects a systematic, uniformly random isotropic plane, the orientation of which is defined by its normal, N_p, from the algorithm shown to the right. Phi, ϕ, is randomly rotated around the z-axis in the interval 0-2π, and theta, θ, is an arcosine weighted angle in the interval 0-π/2. (Modified from Fig. 1 in Larsen et al., 1998).*

Right *A graphic representation of the algorithm used for sampling systematic, isotropic directions of the normal. The position of the first direction is uniformly random within the hemisphere (shown in cylindrical projection), and the succeeding directions are selected systematically with respect to the first. The first ten directions are encircled, direction 11-20 are shown by hexagons, and directions 21-30 by squares (Modified from Fig. 7 in Larsen et al., 1998).*

An equal expected sampling area of virtual planes for all orientations, regardless of the x, y, z-dimensions of the volume probe is achieved with the Cavalieri's principle (Gundersen *et al.* 1988) by exhaustively "sectioning" a fixed volume probe of volume, **v(box)**, with systematic, uniformly random virtual planes with a fixed user defined plane separation distance, ***d***. The position of the first plane is random within an interval equal to the fixed plane separation distance and the succeeding planes are systematically displaced by the fixed plane separation

distance with respect to the previous plane. The expected area of the virtual planes, **E[a(planes)]** within the volume probe is thus constant:

$$\mathbf{E[a(planes)] = v(box)/d.} \quad (1)$$

The intersections between the linear structure and the virtual plane are counted with columns of 2D-disectors (Gundersen 1986) or by using the associated point counting rule (Miles 1978) both with bilateral and lower guard areas. The polygonal virtual planes resulting from the Cavalieri sectioning precludes counting in the well known unbiased counting frame (Gundersen 1977). Fig. 3 and 4 summarizes the counting rule, which is described in detail by Larsen *et al.*, 1998.

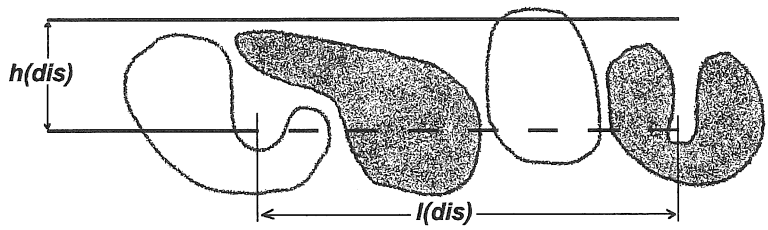

Figure 3
*The 2D-disector counting rule. The 2D-disector comprises an inclusion line (dashed line) of length, **l(dis)**, with an exclusion extension (lower solid line) separated from an infinite look-up exclusion line (upper solid line) by **h(dis)**. The profiles that exclusively touch the inclusion line are counted (heavily drawn) and profiles that touch the look up exclusion line or the exclusion extension are excluded from the count. Note that the heavily drawn profile shown to the left extend into the area between the two exclusion lines but without touching the exclusion lines is included in the count. The 2D disector counting rule ensures correct enumeration of profiles that belong to area **h(dis)• l(dis)** by imposing an unambiguous 2D lexicographic order of the profiles.*

From a uniformly random series of thick physical sections analyzed in systematically sampled, uniformly random microscope fields, an unbiased estimate of the global length density, L_V, is obtained from the classical stereological formula

$$L_V := 2 \bullet \frac{\Sigma Q}{\Sigma a(planes)} \quad (2)$$

where ΣQ is the total sum of the number of transects of the linear 3D structure with the virtual planes inside the volume probes, and $\Sigma a(planes)$ is the total sum of the sampling plane areas (Hennig 1963). Both sums are over all uniformly sampled sections from the reference space. The total length, L, of the linear structure is estimated as:

$$L := L_V \bullet V(ref), \quad (3)$$

where $V(ref)$ is the total volume of the reference space, or directly using a fractionator sampling scheme as described below.

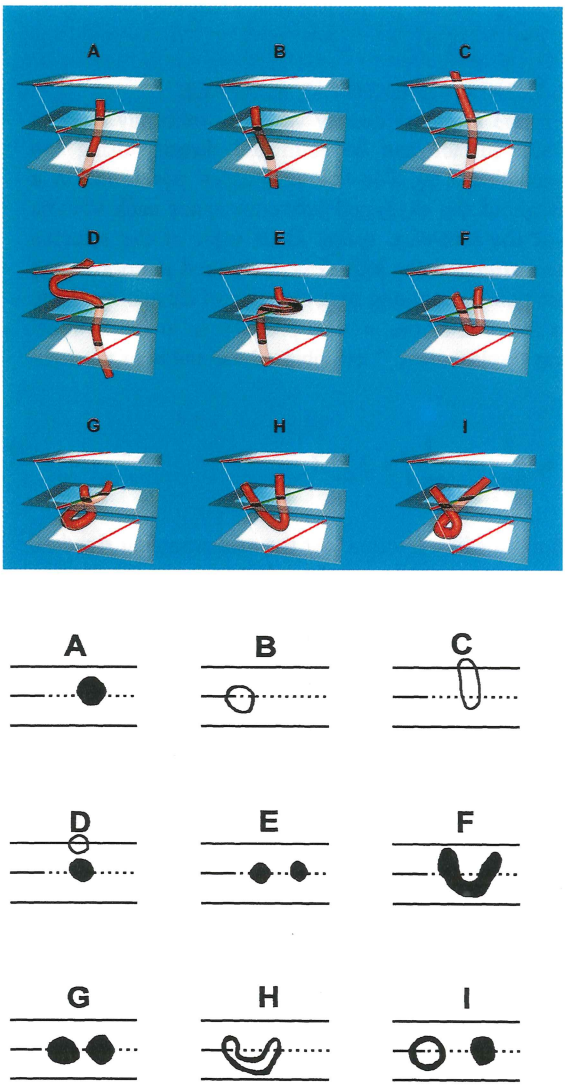

Figure 4

The 2D-disector counting rule in the tilted virtual plane exemplified by various events. Three successive focal planes are shown. The upper focal plane contains the look up exclusion line (red) and a white line showing the projected position of the next disector inclusion line. The middle focal plane contains the inclusion line situated inside the sampling box (green) with the exclusion extension (red, towards the observer), and the line of indifference (blue) that is the 2D representation of the guard area to the right of the 2D-disector. The lowermost focal plane contains the line representing the look down guard area below the sampling box (red). The 2D representation of the structure seen in the focal planes is shown in shadowed red, and the part of the structure between the focal planes is red. The extent of the linear structure in the virtual plane is shown for each event in the sketches below. The ***h(dis)*** *must be small enough that all events are recognized but is made large in the figure for illustrative purposes. One profile is counted in* ***A*** *where the profile exclusively intersects the green inclusion line but none in* ***B*** *where the profile touches the red exclusion extension. No counts in* ***C*** *where the profile is intersected by the look up exclusion line* ***and*** *stays in touch with the moving line during focusing, i.e. remains in the virtual plane, whereas one profile is counted in* ***D*** *where the profile intersected by the look up exclusion line leaves the line during focusing, but is intersected again by the inclusion line. In* ***E*** *the structure is transected twice by the virtual plane as the profiles are connected outside the moving line, i.e. two profiles are counted in* ***E****.*

When multiple profiles located on the inclusion line are disconnected in the focal plane information from the guard area in succeeding focal planes are needed in order to determine whether the profiles represents one coherent structure in the virtual plane. One profile is counted in ***F****, as the two profiles located on the inclusion line stay in touch with and merge on the moving line. Two profiles are counted in* ***G*** *as the two profiles located on the inclusion line merge outside the moving line, i.e. outside the virtual plane. No profiles are counted in* ***H*** *where the profile located on the inclusion line and the profile located on the exclusion extension stay in touch with and merge on the moving line in*

succeeding focal planes. One profile is counted in I where the profile located on the inclusion line and the profile located on the exclusion extension merge outside the moving line. Modified from Fig. 4, Larsen et al. (1998).

FRACTIONATOR DESIGN FOR TOTAL LENGTH AND TOTAL SURFACE

The principle of having a constant probe sampling density within a uniformly random volume probe implies that total length and surface can be estimated directly using the fractionator approach (Gundersen 1986, equation 2.9). For volume probes positioned in a systematic, uniformly random manner throughout the examined reference space each volume probe represents a well defined fraction of the reference space: Over each of the selected physical sections, a volume probe of volume ***a(box)*** times ***h(box)*** is positioned at coordinates of a lattice of systematic, uniformly, random points separated by a distance of ***dx*** and ***dy*** in the x- and y-directions, respectively. ***a(box)*** is the area of the volume probe seen in the xy-focal plane and ***h(box)*** is the extent of focal plane displacement. The unbiased estimator of the total structural quantity, T (L or S) is

$$T := \frac{1}{ssf} \bullet \frac{1}{asf} \bullet \frac{1}{hsf} \bullet \frac{1}{psd} \bullet 2 \sum Z, \tag{4}$$

where T is L or S, Z is Q or I, ***ssf*** the ***s***ection ***s***ampling ***f***raction, ***asf*** the ***a***rea ***s***ampling ***f***raction, ***hsf*** the ***h***eight ***s***ampling ***f***raction, and ***psd*** the constant ***p***robe ***s***ampling ***d***ensity. ***ssf*** is the fraction of uniformly sampled sections from the reference space, e.g. 1:10. ***asf*** is ***a(box)/dx•dy***, ***hsf*** is $h(box)/\bar{t}$, where $\bar{t}$ is the average section thickness

$$psd = \frac{E[a(planes)]}{v(box)} = \frac{1}{d}$$

for virtual planes, where d is the constant plane separation distance

$$psd = \frac{E[l(lines)]}{v(box)} = \frac{1}{d_1 \bullet d_2}$$

for virtual lines, where d_1 and d_2 are the constant line separation distances

Practical examples of the use of isotropic virtual planes for the estimation of L_V and L of microvessels in the rat cerebellum and for regenerating neurofilament-positive fibres in rat spinal cord lesions can be found in Larsen *et al.* (1998) and vonEuler *et al.* (1998), respectively.

The described test system can also be used for local spatial sampling in uniformly sampled particles for estimating local length, l, of structures completely contained within the thick section.

DISCUSSION

The advantages of virtual test systems are manifold. For all global estimators there is complete freedom to choose the most convenient sectioning direction. As a consequence, estimation of orientation dependent parameters from tissue regions where a specific section plane is needed is for the first time possible, and arbitrarily orientated, uniformly, random thick sections originally made for other purposes can now be used for quantitative analyses. In addition, virtual test probes provide optimal conditions for reducing estimator variance in studies where a high magnification is required. Reduction of the estimator variance from estimation of strongly anisotropic

structures is for most estimators best obtained by probing the structure with many different test probe orientations. Large numbers of probe orientations in the volume probes can easily be obtained with virtual test probes, whereas an equivalent number of different physical sectioning directions will be extremely labour intensive. In terms of efficiency it should be mentioned that the virtual test system depend on high power immersion objectives (see below). For estimation of global length and surface of a structure that can be analyzed at a low magnification it may in some cases be more efficient to study few orientations at physical sections which make available large areas for counting at a low magnification, rather than counting from many directions at a high magnification, i.e. on a small area.

Other virtues of the virtual test systems include the possibility to estimate length and surface directly using a fractionator design, and the potential for direct estimation of directional distributions (Mathieu *et al.*, 1983) from sets of parallel sections. Finally, the estimators are unaffected by deformation, and give a more certain recognition of the structure of interest due to the possibility of obtaining information from more focal planes.

For using virtual test systems it is mandatory to use equipment capable of integrating correct z-axis displacement with the software. Also, one must be able to make relatively thick physical sections, and to visualize the structure of interest with stains that penetrates the thick physical sections. Other disadvantages are that high-power immersion objective providing thin focal planes has limited working distance, and that observations and decisions for the counting rule are not made in only one focal plane.

Obviously, dimensional changes of the examined structure induced during preparation of the structure introduce bias in the estimate, the ratio estimators being sensitive to differential changes in the structure(s) and their reference space, whereas the straightforward fractionator estimators are only sensitive to changes of the structure itself. For estimators based on symmetric counting rules, as e.g. the 2D-disector, early respectively late recognition results in a unidirectional shift in detection plane and is unbiased (see West *et al.* 1991 for a detailed description of early respectively late recognition).

The principle of 3D randomization of a virtual test system and subsequent counting in a 2D representation of the test system during focal plane displacement has potential for all global stereological relationships, and provides optimal conditions for the quantitative study of orientation dependent parameters. The use of virtual test systems for local spatial sampling in uniformly sampled particles is under current investigation.

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